reaction 7 (Webb, 1966), which is 13.22, we obtain log K_8

$$Hg^{2+} + 2Cl^- \rightarrow HgCl_2 \tag{7}$$

for reaction 8 as 28.91. The log K_9 for reaction 9 (Gould

$$Hg^{2+} + 2(C(=O)NH^-) \rightarrow Hg(HNC(=O))_2 \tag{8}$$

and Sutton, 1970) is 30.2, so that subtracting eq 9 from

$$2(C(=O)NH^-) + 2H^+ \rightarrow 2(C(=O)NH_2) \tag{9}$$

eq 8 we finally obtain:

$$Hg^{2+} + 2(C(=O)NH_2) \rightarrow Hg(HNC(=O))_2 + 2H^+ \tag{10}$$

and log $K_{10} = -1.3$. For 1 mol of amide bound to Hg(II) we can take the square root as a good approximation and have log $(K_{10})^{1/2} = -0.65$. The major uncertainty in this result comes from the use of log K_9 which might vary by one unit to either side of the reported value.

This calculation suggests that the two types of groups, i.e. COOH and CONH$_2$, should be distinguishable by Scatchard analysis since the ratio of the K values is about 1000:1. No distinction is apparent from the experimental data. In addition, neither site by itself yields a K which is large enough to compare with the experimental values of $>10^4$. Indeed, even larger intrinsic constants for the association of Hg(II) with the proteins are to be expected since the charge on both proteins is high at this pH, +18 for lysozyme and +14 for ribonuclease A.

We conclude that the carboxylic acid and carboxylic acid amide groups cannot together provide a single class of sites for the binding of Hg(II) ion to either protein. It is known from the study of model systems (Kamenar and Grdenic, 1969) that mercury(II) ion forms two strong bonds with ligands and may also form one or more weaker ones. Therefore, a likely possibility is that the binding sites for Hg(II) ion are not monofunctional, i.e., consisting of a single carboxylic acid or a single amide group. Instead, each site may be comprised of two or more ligands. These

may be the aforementioned side-chain carboxyl and amide groups and backbone peptide linkages. Indeed, even the aromatic side-chain groups could participate since benzene is known to be mercurated (Westheimer et al., 1947) in aqueous nitric acid solutions at slightly more elevated temperatures. Steric arrangements of the ligands consistent with the primary structure of the protein will determine which groups on the protein are involved in the binding. Further work is required to better characterize the nature of these sites.

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Preparation of Chromium-Containing Material of Glucose Tolerance Factor Activity from Brewer's Yeast Extracts and by Synthesis

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When Brewer's yeast was extracted with dilute alcohol and purified by ion exchange chromatography the resulting preparations were shown to have glucose tolerance factor (GTF) activity. They potentiated the action of insulin on the glucose oxidation of chromium-deficient rat adipose tissue in vitro. Such preparations were found to contain chromium, nicotinic acid, glycine, glutamic acid, and cysteine. Reacting trivalent chromium with these ligands in vitro yielded a mixture of chromium complexes which exhibited GTF properties, similar to the material separated from Brewer's yeast.

The glucose tolerance factor (GTF) contains the essential trace element, chromium (Mertz, 1969). This compound (or group of compounds) occurs in Brewer's yeast and other foods (Toepfer et al., 1973); its exact structure is yet unknown. Chromium as part of GTF is more potent in chromium-deficient animals than are simple chromium compounds, such as tetra- or hexaaquo

complexes. The best known function of GTF is the potentiation of the action of insulin on chromium-deficient tissue (Mertz and Roginski, 1971). Not all chromium in biological materials has GTF activity, as the latter is not significantly correlated to the *total* chromium content of different materials. There is, however, a significant correlation between biological function and that part of the total chromium which is extractable by 50% ethanol (Toepfer et al., 1973). Brewer's yeast was found to be outstanding among all foods tested in that approximately one-third of its high chromium content was in this form;

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it was therefore considered the best source of starting material for the concentration of GTF. This publication will describe a procedure to extract and concentrate GTF from Brewer's yeast and gives a method to prepare chromium complexes which are similar to purified yeast preparations not only in chemical behavior but also in their biological effect.

Even though these procedures do not result in isolations and unequivocal structural determinations of one or more compounds, they are reported here because they represent the first successful attempt to synthesize substances with biological GTF activity similar to naturally occurring fractions.

EXPERIMENTAL SECTION

Biological Activity. The relative biological activity of the preparation was determined in vitro using chromium-deficient rat epididymal adipose tissue. The assay, which has been described in detail (Mertz, 1969; Roginski et al., 1970), compares the slope of the tissue reponse to graded levels of insulin in the presence of GTF to that obtained without GTF. For most routine assays, however, only one insulin concentration was used, at least in triplicate assays. The term "relative biological activity" denotes the factor by which the added GTF preparation increased the insulin effect on the tissue.

Extractions of Brewer's Yeast. Brewer's yeast (*Saccharomyces carlsbergensis*) was either grown in the laboratory in Sabouraud's medium containing chromium or purchased commercially.

The extraction of Brewer's yeast was carried out in the laboratory by suspending 1 kg in 6 l. of 50% ethyl alcohol, with subsequent heating just to boiling and cooling to room temperature overnight. After filtration, the alcohol was removed in vacuo and the remaining aqueous solution was treated with 250 g of activated charcoal (Darco G-60) at pH 3.5 (HCl) for 1 h and filtered. The charcoal cake was washed first in 1 l. of 95% ethyl alcohol, and then in 1 l. of water; all the filtrates were discarded. The material containing biological activity was eluted from the charcoal by suspending it in 2 l. of a mixture (1:1) of concentrated NH$_4$OH and diethyl ether for 3 h, followed by filtering and washing. The step was repeated once; then the ammonia and ethyl ether were removed in vacuo. This procedure produced consistent results. Although it was more efficient on a laboratory scale than were extractions on a pilot plant scale, extracts produced by a pilot plant were routinely used as starting material for subsequent steps.

Material extracted from 1 kg of yeast in 25 ml of aqueous solution (100 mg dry weight/ml) was hydrolyzed by refluxing for 18 h with 5 N HCl. After removal of HCl in vacuo the hydrolyzed material was extracted with ether and finally made to the original volume at pH 3.0.

The most successful purification procedure of the extracted material was ion exchange chromatography with Dowex 50W-X8 cationic resin. The resin was prepared by washing with 0.1 N NH$_4$OH, water, 0.1 N HCl, and water. The column provided 17 mm × 350 mm of resin; the elution rate was usually 1 ml per min. The acid-hydrolyzed solution was applied to the column and was washed with water to remove salts and other extraneous materials. The absorbed compounds were eluted with 0.1 N NH$_4$OH and monitored at 262 nm. A broad peak was observed at this wavelength (Figure 1). The fractions were of orange-red color and all contained chromium and distinct biological activity. They were combined for further separation procedures.

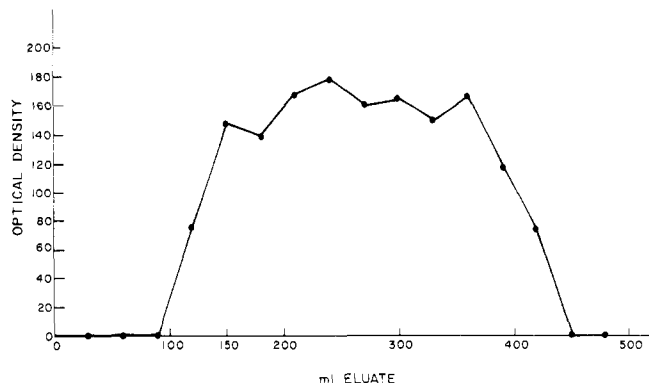Subsequently, the colored material was passed through successive ion exchange columns. Although much ex-

Figure 1. Yeast extract chromatogram from Dowex 50W-X8 column.

traneous material was removed through the third column, as shown by the increasing ratios of chromium to dry weight, glucose tolerance factor activity was not any further concentrated by this procedure.

The material from the ion exchange columns was found to contain nicotinic acid as deduced from the presence of a pyridine structure in data from mass spectrographs and the previously observed peak in optical density scans at 262 nm. Nicotinic acid was also sublimed from this material and was extracted by organic solvents for the purposes of identification. Amino acid analyses showed glycine, glutamic acid, and cysteine as well as trace amounts of other amino acids.

Synthesis of Chromium Complexes. Chromium complexes were prepared by dissolving 4 g of Cr(Ac)$_3$·H$_2$O, equivalent to 840 mg of Cr, in 750 ml of 80% alcohol containing 2 ml of glacial acetic acid that was neutralized with NH$_4$OH to pH 7. Four grams of nicotinic acid (molar equivalent 2:1 Cr) was added to the contents of the flask and was stirred during refluxing for 3 h until a distinct color change occurred. Successively, 2.4 g of glycine (2:1 Cr), 2.4 g of L-glutamic acid (1:1 Cr), and 2.5 g of L-cysteine-HCl (1:1 Cr) were added during 4 h of continuous stirring and refluxing. The material was stirred overnight without being heated. Alcohol was removed in vacuo, the solution was filtered (Whatman No. 40) to remove suspended insoluble material, the residue was washed with water, and filtrate and washings were combined and reduced in vacuo to 300 ml. This solution of crude material was a deep red color at pH 4.3. Using the procedure previously described for a smaller column, 150 ml was passed through a 5 cm × 55 cm Dowex-50 column. When freeze dried, material eluted from this column was hygroscopic. If neutralized with KOH to obtain presumably the K salt, the material became less hygroscopic with no change in other properties. With this purification step through Dowex-50, the first material through the column was highly colored but had no absorption at 262 nm and no biological activity. Only the material eluted with NH$_4$OH showed the desired properties. Hence, washing the column with water removed salts, acids, and water-soluble materials and permitted some degree of purification.

Properties of Synthesized Material. Synthesized materials from Dowex-50 columns have a definite peak at 262 nm with slight shoulders showing between 250–260 nm and 260–270 nm, very much like the material isolated from Brewer's yeast. Undoubtedly, the presence of nicotinic acid in the complex was responsible for the 262-nm peak. When the synthesized material was assayed in the in vitro system for its potentiation of insulin action, a high response was observed with amounts corresponding to nanograms
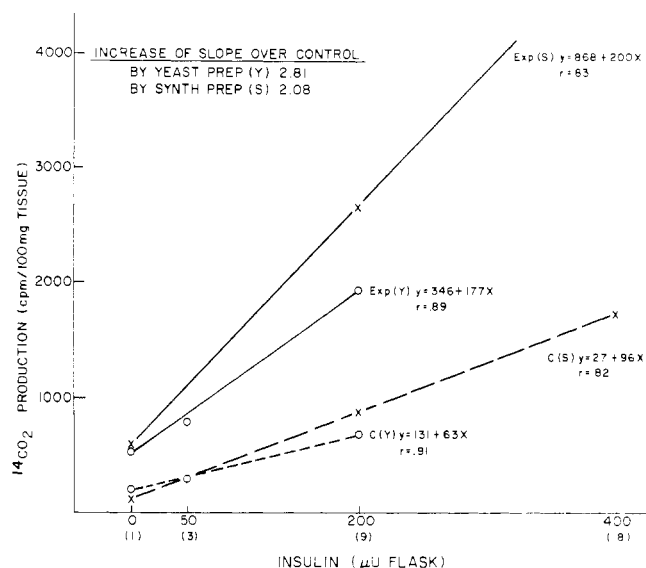
Figure 2. Insulin dose response for yeast and synthetic preparations as determined by CO_2 from glucose oxidation. The response of fat tissue to increasing concentrations of insulin is not linear; also, the term "zero insulin" includes an uncertain amount of insulin-like activity present. The regression lines were drawn between the points for "zero insulin" and the highest insulin concentrations and intermediate insulin concentrations were assigned a value according to best fit. Zero, 50, 200, and 400 μU are represented by the numbers 1, 3, 9, and 18, respectively; these latter values have been used for the calculation of the regression.

of Cr/ml of incubation medium. Complexes of chromium with nicotinic acid alone (presumably of a dinicotinato, tetraaquo configuration) gave a measured degree of biological activity, immediately after having been prepared. They were, however, unstable and precipitated at near neutral pH, resulting in the loss of biological activity. Complexes of chromium and nicotinic acid with other amino acids were easily prepared; their biological activity was variable and less than that of the complexes described above. Prior to use of the ion exchange column with synthesized material, the linear relationship between chromium (1.0 to 2.8 μg per ml) and optical density was found to be $y = 0.2190 + 0.1625x$. Such material was found to contain 5.39% Cr dry weight. After ion exchange the relationship was found (at 0.1 to 1.7 μg of Cr per ml) to be $y = 0.2643 + 0.394x$; with $F(1/26) = 99.77$, the regression was highly significant ($p < 0.01$). The linear equation for the potassium salt showed almost the identical slope, $y = 0.4197 + 0.390x$.

Table I shows the compositional analyses of one of the synthetic preparations after purification by ion exchange chromatography. Ammonia nitrogen was subtracted from total nitrogen to indicate the bound nitrogen. The components for which analytical data were obtained (chromium, nicotinic acid, glycine, glutamic acid, cysteine, and NH_3) accounted for almost 88% of the samples.

Comparison of the Properties of Yeast Preparations with Those of Synthetic Material. The similar action of the natural and the synthetic material in the biological assay suggested, but did not prove, a similarity of chemical structure (Figure 2). Therefore, additional information was sought as to the physical and biological characteristics of the compounds, when both categories were subjected to identical separation procedures.

The pattern of biological activity of Dowex-50 eluates of yeast (after charcoal treatment) was very similar to that of the synthetic material (Figure 3). The biological activity
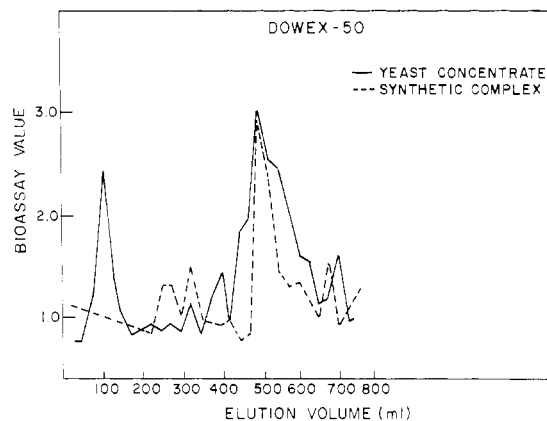

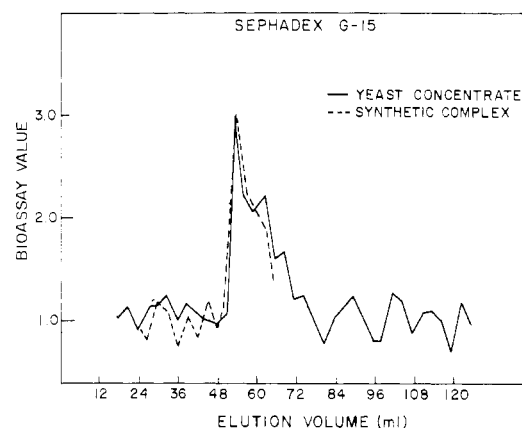Figure 3. Comparison of chromatograms of yeast concentrate and synthetic complex from Dowex-50.

Figure 4. Comparison of chromatograms of yeast concentrate and synthetic complex from Sephadex G-15.
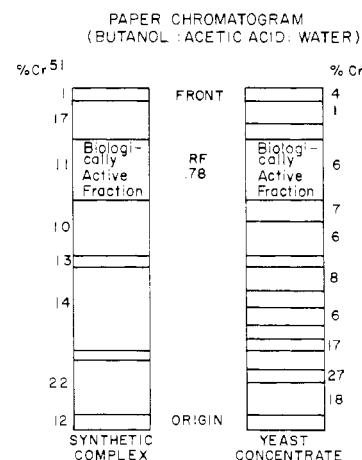

Figure 5. Paper chromatograms of yeast concentrates and synthetic complex.

in the initial Dowex-50 fractions of the yeast extract was not duplicated in the synthetic material, but this initial peak was not consistently observed. The two categories were again compared in a different chromatography system using Sephadex-15 columns. Here again, the elution patterns were nearly identical (Figure 4).

A third comparison was made by using ascending paper chromatography (Whatman No. 1, 1-butanol, acetic acid, and water). The major bands were identified under UV light, cut apart, and eluted with water. The biological assay demonstrated the main biological activity in a band with an R_f of 0.78, for both the natural and the synthetic material. The distribution of chromium, measured by γ

Table I. Composition of Synthesized
Chromium Complex

Component	%	µg mol/mg	Molar ratio to Cr
Chromium	5.66	1.09	
Total nitrogen	16.46		
NH₃ nitrogen	8.96	5.27	4.8
Bound nitrogen	7.50		
Nicotinic acid	28.17	2.29	2.1
Glycine	14.70	1.96	1.8
Glutamic acid	17.64	1.20	1.1
Cysteine	13.20	1.10	1.0
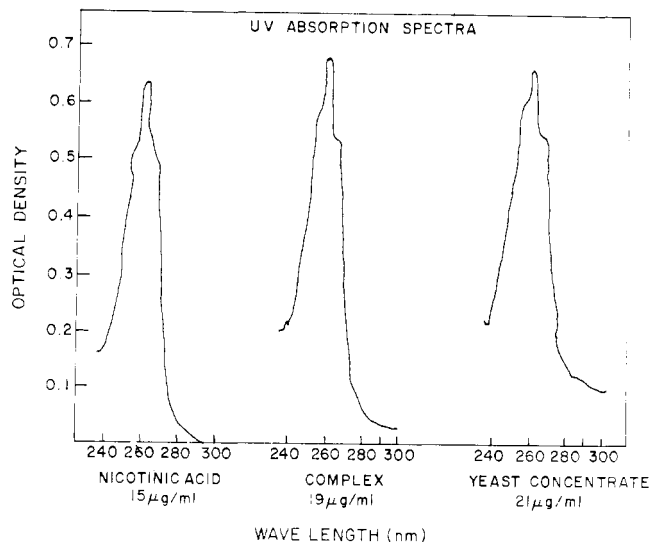
Figure 6. Ultraviolet absorption spectra of aqueous solutions of nicotinic acid, synthetic complex, and yeast concentrate.

counting of ^{51}Cr in the synthetic fractions and by flameless atomic absorption spectrometry in the yeast preparations, indicated a number of different chromium compounds, with less or no biological activity. The chromium in the band with the major biological activity represented 11 and 6% of the total, for synthetic and natural preparations, respectively (Figure 5).
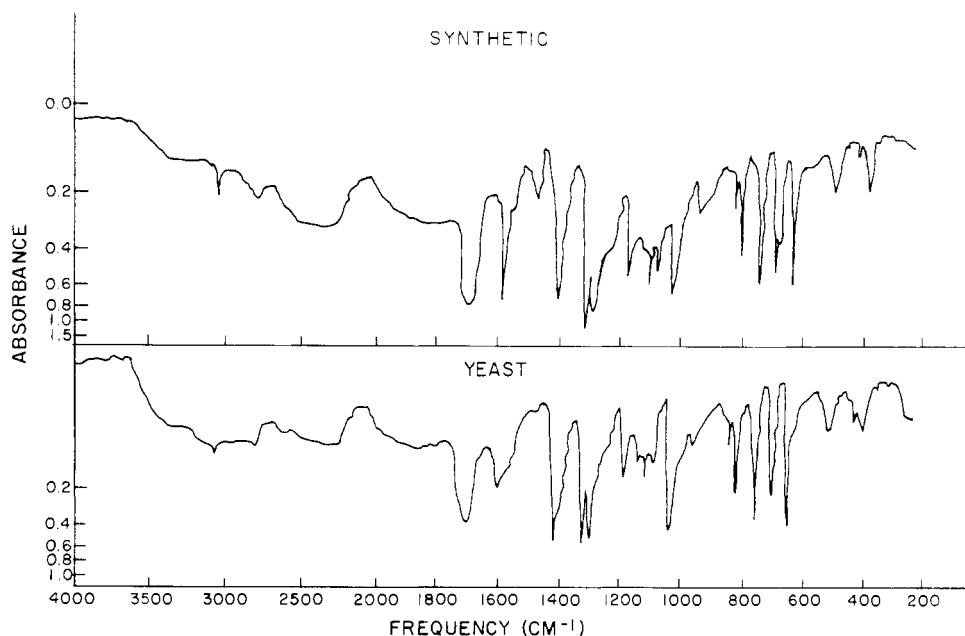
The UV absorption spectra of these materials, concentrated from yeast or synthesized, obtained with a Cary 14 recording spectrometer were almost identical, with maxima at 262 nm (Figure 6). Shoulders on each side of the 262-nm peak were typical for both natural and synthesized material and were not as pronounced in pure solutions of nicotinic acid.

Infrared spectra (Figure 7) of both yeast concentrates and synthesized material showed typical absorptions for nicotinic acid and chromium as well as rather broad absorptions for -COOH groups of the amino acids. Again, a remarkable degree of similarity between the spectra is evident.

DISCUSSION

The significance of these findings lies in the fact that for the first time a group of compounds has been synthesized and partly identified that possesses biological activity similar to the naturally occurring chromium compounds in yeast. Their nutritional and biological implications have been discussed elsewhere (Mertz et al., 1974).

The definitive identification of glucose tolerance factor in yeast has not yet been accomplished. This would require the isolation in crystalline form of sufficient amounts of the factor for structural identification. In view of the low concentration of GFT in yeast and other material and taking into account its relative instability, it may be especially difficult to identify its structure by isolation from natural materials. But this may not be necessary. Chromium as part of GTF differs so distinctly from other chromium compounds in its biological activity that the latter is a valid criterion to assess the success or failure of the synthetic approach. The similarity of biological, chemical, and physical characteristics of the natural and the synthetic preparations strongly suggests, but does not prove, the niacin–chromium–niacin axis as the essential GTF configuration. The optimal composition of the amino acid ligands is unknown, as is the functional group on the nicotinic acid to which chromium is coordinated. Furthermore, even the best synthetic preparations described here are impure and probably consist of a mixture of chromium complexes and isomers which have not yet been resolved.

Figure 7. Infrared spectra of yeast concentrate and synthetic complex.

Much work remains to be done before the exact structure can be identified, but the final identification promises to be an important step toward the establishment of the chromium requirement and the assessment of the chromium nutrition in man.

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Heat-Induced Complex Formation between Myosin and Soybean 7S Globulins

Neville L. King

Myosin and β-conglycinin (a soybean 7S globulin fraction) were found to associate after exposure to temperatures in the range 75–100 °C. The complexes formed sedimented more slowly, and their subunit compositions were different from those of aggregates derived from β-conglycinin heated alone. The sedimentation coefficient of the β-conglycinin aggregate increased with incubation temperature, and its subunit composition became progressively richer in the component of lowest molecular weight. At lower temperatures (2 and 25 °C) no evidence for any complex formation was obtained when mixtures of myosin and β-conglycinin in phosphate buffer (pH 7.6, ionic strength 0.5) were examined by viscometry, gel chromatography, and density gradient centrifugation.

Changes in texture induced by the incorporation of soy proteins in manufactured meat products have been reported to be large in relation to the amount of soy protein added (Hermansson, 1973). It is possible that interactions between the proteins of muscle and soybeans might contribute to these changes. In view of the difficulties in examining whole meat products for the presence of interactions, solutions and suspensions of protein fractions from muscle and soybeans have been employed as model systems. Yamamoto et al. (1973) examined the 11S globulin and acid-precipitated proteins from soybeans in admixture with myosin and actomyosin. They detected no evidence for interactions at low temperatures, but found, by turbidimetric measurements, that myosin and soy protein aggregated to a lesser extent when heated in combination, than when heated separately.

In order to obtain further information on the above model systems, mixtures of the major protein components of muscle and soybeans are being investigated by alternative techniques. The present article deals mainly with the sedimentation properties and subunit composition of products formed from myosin and β-conglycinin after heating. β-Conglycinin is one of the two immunochemically distinguishable forms of soybean 7S globulins (Catsimpoolas and Ekenstam, 1969).

Changes in quaternary structure of proteins give rise to difficulties in the nomenclature used to describe the products formed (e.g., see Joly, 1965). For convenience, the term "aggregate" will be used here to describe a high molecular weight product derived from components of the same protein fraction, the term "complex" will be employed for products incorporating components of different protein fractions, while "interaction" will be used more widely to include effects such as hydrodynamic interactions which result in changes in physical properties but not necessarily in quaternary structure.

MATERIALS AND METHODS

Phosphate buffer consisted of: 0.035 M potassium hydrogen phosphate, 0.4 M sodium chloride, and 0.02% sodium azide, pH 7.6.

Myosin. Myosin was prepared from longissimus dorsi muscles of rabbits by the extraction and ammonium sulfate fractionation procedures of Offer et al. (1973). The content of contaminating proteins (see, e.g., Offer et al., 1973; Pinset-Harstrom and Ehrlich, 1973) was reduced by using the additional purification steps recommended by Wikman-Coffelt et al. (1973). Finally, myosin was dialyzed against phosphate buffer and centrifuged at 30 000 rpm for 0.5 h and the supernatant ($\sim$10 mg/ml) was stored at 2 °C.

β-**Conglycinin.** Soybean 7S globulins were prepared by the following procedure, based on that of Roberts and Briggs (1965). Defatted soy flour was extracted with water (5 ml of water/g of flour) by stirring for 1 h at room temperature. After centrifuging at 20 000g for 15 min at 15 °C, the supernatant was passed through glass wool and allowed to stand overnight at 2 °C. The precipitated material (mainly 11S globulin) was removed by centrifuging at 0 °C. The supernatant was subjected to ammonium sulfate fractionation; the material precipitating between 0.80 and 0.95 saturation was dissolved in phosphate buffer (1 ml/g of soy flour) and the ammonium sulfate fractionation was repeated. The 0.80–0.95 fraction, after dissolving in phosphate buffer as above, was adjusted to pH 4.5 with acetic acid and dialyzed against water to precipitate the 7S globulins. After centrifuging, the precipitate was dissolved in phosphate buffer to give a

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